American Journal of Hematology 54:53–60 (1997)

Inherited Thrombotic Disorders: An Update

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Significant advances in identification of etiologies of inherited thrombosis have been recently reported. A point mutation in coagulation factor V (factor V Leiden) results in resistance to activated protein C and probably repesents the most common genetic risk factor for venous thrombosis. A metabolic disorder, homocysteinemia, is now known to be an important risk factor for both arterial and venous thrombosis. Many patients with recurrent thrombosis will have more than one genetic risk factor identified. Recognition of these new disorders should permit a diagnosis to be achieved in at least half of patients evaluated for inherited thrombosis. Am. J. Hematol. 54:53–60, 1997 © 1997 Wiley-Liss, Inc.

Key words: activated protein C resistance; homocysteinemia; factor V

INTRODUCTION

The past few years have witnessed significant advances in our knowledge of inherited thrombotic disorders. A few years ago, up to 70% of thrombosis in patients with appropriate clinical features for inherited thrombosis and no identifiable risk factors (malignancy, vascular disease, etc.) were termed "idiopathic." Now, molecular diagnostics combined with existing laboratory techniques allow accurate classification of at least half of patients with inherited thrombotic disorders.

The most significant advance has been identification of activated protein C resistance (APC-R) as a common inherited thrombotic disorder. This disorder was initially described by Dahlback in 1993 [1] and may account for up to 52–64% of inherited thromboses [2]. Another important advance is the appreciation that a disorder previously thought to be associated with arterial thrombosis, homocysteinemia, may also be a common etiology for venous thrombosis [3,4]. Table I summarizes the inherited thrombotic disorders, the mechanisms responsible for thrombosis, prevalence, and clinical features of the disorders.

The purpose of this review is to briefly summarize advances on clinical and laboratory aspects of inherited thrombotic disorders. This review will focus on new information published in this area since 1992, with particular emphasis on APC-R and homocysteinemia.

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ACTIVATED PROTEIN C RESISTANCE

Activated protein C resistance (APC-R) is characterized by a poor anticoagulant response to APC. Protein C is a vitamin K-dependent serine protease activated by the thrombin-thrombomodulin complex on the endothelial cell surface. Once activated, the enzyme degrades activated clotting factors Va and VIIIa [5]. The molecular defect, a point mutation at nucleotide position 1691 of the factor V molecule (factor V Leiden), substitutes glutamine for arginine at the APC cleavage site [6], altering the factor V molecule such that it no longer is degraded by APC, yet still retains its procoagulant activity, favoring thrombosis. Although the affected cleavage site in APC-R is not directly responsible for inactivation of factor Va, cleavage at this site is required for subsequent proteolysis. Cox et al. [7] provided evidence that the mutation may be traced back to a single European founding population, and that the prevalence is high because it conferred some undetermined selective advantage. Moreover, this muta-

Contract grant sponsor: VA Research Office.

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Received 1 July 1996; Accepted 7 August 1996

TABLE I. Summary of the Inherited Thrombotic Disorders*

Classification and disorders	Mechanism for thrombosis	Inheritance[a]	Estimated prevalence (%)	Clinical features
Deficiency or qualitative abnormalities of activated coagulation factors				
Factor V Leiden (APC-R)	APC fails to inactivate factor Va due to highly conserved point mutation	AD	20–60	Venous thromboembolism
Protein C deficiency	Failure to generate APC; failure to inactivate factors Va and VIIIa	AD	5–6	Venous thromboembolism
Protein S deficiency	Failure of APC to inactivate factors Va and VIII	AD	5–6	Venous thromboembolism
AT III deficiency	Failure to inhibit thrombin, factor Xa, and other activated factors	AD	1–2	Venous thromboembolism (usual and unusual sites), heparin resistance
Heparin cofactor II def.[b]	Failure to inhibit thrombin	AD	<1	Venous thrombosis
Impaired clot lysis				
Dysfibrinogenemia	Abnormal fibrin resists fibrinolysis	AD	1–2	Venous thrombosis > arterial thrombosis
Plasminogen deficiency	Failure to generate plasmin	AD (AR)[c]	1–2	Venous thromboembolism
t-PA deficiency	Failure to activate plasminogen	AD	?	Venous thromboembolism
Excess PAI-I activity	Neutralization of t-PA	AD	?	Venous thromboembolism and arterial thrombosis
Metabolic defect				
Homocysteinemia (Homocystinuria)	Endothelial cell cytotoxicity and perturbation of vascular hemostatic mechanisms	AR	1 in 250,000	Homozygous patients have physical abnormalities in addition to arterial and venous thrombotic disease and accelerated atherosclerosis
			25% of pts. with recurrent thrombosis, 10% of pts. with first episode of thrombosis	Heterozygous patients develop premature arterial thrombotic disease (coronary, cerebral, peripheral) as well as venous thromboembolism

*Adapted from Rodgers and Chandler [33].

[a]AD, autosomal dominant; AR, autosomal recessive.

[b]Whether heparin cofactor II deficiency represents a positive risk factor for thrombosis is uncertain.

[c]Quantitative plasminogen deficiency is usually inherited as an AD disorder, while dysplasminogenemia is inherited as an AR disorder.

tion is rare in non-European populations [7]. In its heterozygous form, the defect increases the carrier's risk of thrombosis by up to 7-fold [8]. The expression of the phenotype is highly variable, and generally is not as severe as inherited protein C or protein S deficiency [9,10]. However, it is clear that non-genetic factors such as surgery, pregnancy, and the use of oral contraceptive agents increase the risk of venous thrombosis in APC-R patients [9–12]. The risk of thrombosis in homozygous individuals is estimated at 80-fold and most patients will suffer at least one episode of thrombosis during their lifetime [12]. However, Zoller et al. [13] reported that of 18 homozygotes at age 33 years, 60% were asymptomatic, again underscoring the importance of additional genetic or non-genetic defects precipitating thrombotic events. Perhaps the most important non-genetic risk factor is the use of oral contraceptives; homozygous females using these drugs are at significant risk for venous thrombosis [9,10]. There is a notable lack of association of APC-R [14], AT III, protein C, or protein S deficiencies with arterial thrombosis.

Pregnancy is a hypercoagulable state, and thrombosis is a common cause of maternal death [15]. Bokarewa et al. [15] reported that, among 70 Swedish women suffering thrombosis in pregnancy, fully 46% had the factor V mutation, emphasizing the importance of this non-genetic risk factor in females with APC-R.

In well-defined patient populations, the prevalence of APC-R in patients with thrombosis ranges from 13% [16] to 52–64% [2]. One study of APC-R testing in a reference laboratory drawing samples from academic and community hospitals throughout the nation reported a prevalence of 12% [17]. However, the prevalence of APC-R among patients with thrombosis is difficult to ascertain with certainty because of referral and reporting biases. The prevalence of the factor V mutation in the general population ranges from 2–5% among European countries studied [6,8,18]. We assume from these data that the prevalence within the United States is similar; however, a study of the United States population has not been published.

In addition to being associated with venous thrombosis, APC-R is also associated with recurrent miscarriage (loss of three or more consecutive pregnancies) within the second trimester. Although the majority of miscarriages in the second trimester remain idiopathic, 20% are associated with APC-R [19] and 12% with the lupus anticoagulant or anticardiolipin antibodies [20].

One emerging concept from new epidemiologic studies on the inherited thrombotic disorders is that many patients with recurrent thrombosis have more than one genetic risk factor. APC-R is present in 14% [21] to 19% [22] of symptomatic protein C-deficient patients. Zoller et al. [23] reported that among 18 females with hereditary protein S deficiency, 39% (7 of 18) had the APC-R mutation. Moreover, in family members with combined defects, 72% suffered thrombotic episodes, compared with 19% with only the factor V mutation and 19% with only protein S deficiency [23].

Clinical Considerations

Samama et al. [9] suggest that with the current available data, no recommendation can be made for long-term anticoagulation in patients suffering a first episode of thrombosis. Life-long prophylaxis is probably not necessary for individuals that are heterozygous or homozygous for the mutated factor V [9,12], unless they experience more than one thrombotic event or their first thrombotic event is life-threatening. We would recommend, however, that oral contraceptive agents be used with caution in APC-R patients because of the increased risk for thrombosis. Women suffering a second trimester pregnancy loss as well as pregnant females with thrombosis should be investigated for inherited thrombotic disorders, especially APC-R and antiphospholipid antibodies. Controversial issues in this area include whether female patients should be screened for APC-R before initiating estrogen therapy, and whether asymptomatic APC-R female patients who become pregnant should receive anticoagulant prophylaxis.

HOMOCYSTEINEMIA

Homocysteine is an intermediate of methionine metabolism. Figure 1 illustrates the transsulfuration pathway that involves homocysteine metabolism. Elevated plasma levels can result from a deficiency of cystathione β-synthase (CBS) [24], homozygous expression of a thermolabile form of methylenetetrahydrofolate reductase (MTHFR) [25], or from acquired deficiencies of vitamin B_{12} or folic acid required in remethylation of homocysteine to methionine [24].

High plasma levels of homocysteine have been associated with premature atherosclerosis in adults, and have been regarded as a risk factor for arterial thrombosis [24,26]. More recently, mild elevations of homocysteine have also been implicated in the pathogenesis of venous thrombosis [3,4]. den Heijer et al. reported that 25% of patients with recurrent venous thromboembolism [3] and 10% of patients with a first episode of venous thrombosis had homocysteinemia [4]. Although it is unknown why increases in plasma homocysteine lead to pathologic changes, it has been firmly established that patients with homozygous CBS deficiency manifest central nervous system abnormalities, including mental retardation and psychiatric disturbances, skeletal and eye abnormalities, and a tendency to thrombose, usually before the age of thirty [24]. These patients have traditionally been diagnosed under the disease category “homocystinuria,” a rarer condition that should be distinguished from the milder heterozygous state that has recently been associated with venous thrombosis.

In vitro studies indicate that homocysteine reduces the activation of protein C by endothelial cells, stimulates the activation of factor V, and induces endothelial cell tissue factor activity, thus contributing to the thrombotic tendency seen in homocysteinemic patients [27]. However, only one third of such patients develop thrombosis [28]. Mandel et al. [29] propose that this variability could be due to the co-existence of additional inherited thrombotic defects in homocysteinemic patients, especially APC-R. In their study of seven consanguineous unrelated Israeli-Arab families, they observed that major thrombotic events occurred only in patients that were homozygous for homocysteinemia and homozygous or heterozygous for factor V Leiden. Moreover, of sixteen heterozygous carriers for homocysteinemia, none had suffered episodes of arterial or venous thrombosis, even among four patients that were homozygous or heterozygous for factor V Leiden, suggesting that other non-genetic factors may be necessary to induce thrombosis, such as surgery or the use of oral contraceptives. Figure 2

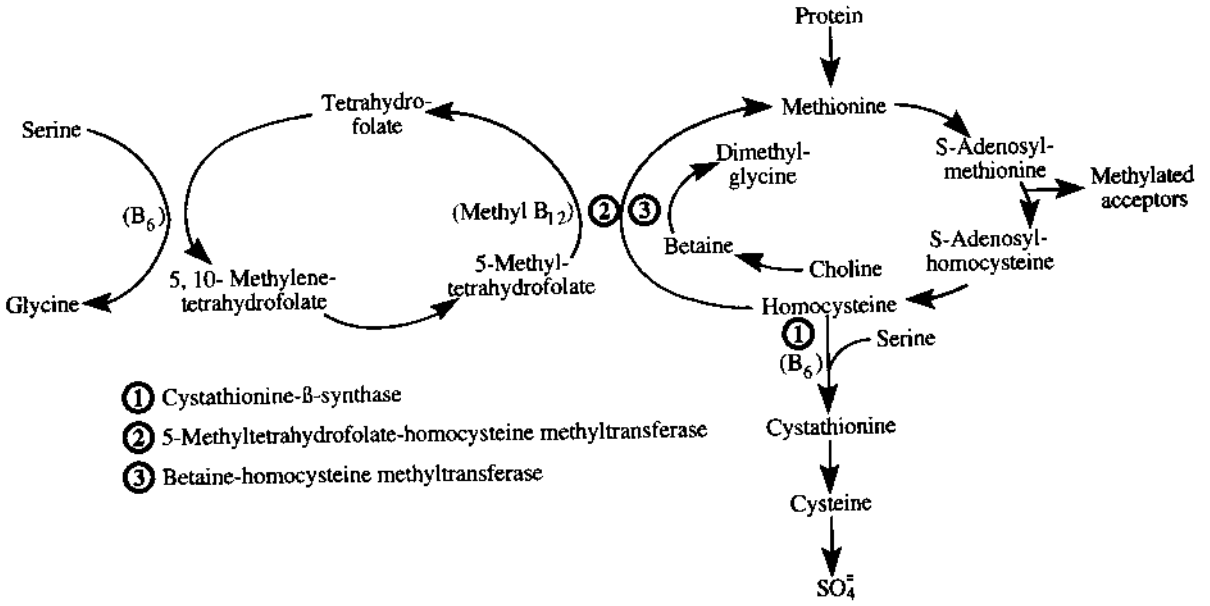

Fig. 1. The transsulfuration pathway. The sulfur atom of methionine is converted into the sulfur atom of cysteine. Associated with the transsulfuration pathway are metabolic reactions in which the methyl group of methionine is transferred to generate a variety of methylated compounds (transmethylation reactions), and the reformation of methionine by methylation of homocysteine. Important regulatory enzymes are depicted. Homozygous and heterozygous homocysteinemia most commonly result from deficiencies of cystathionine-β-synthase activity. Reprinted from Rees and Rodgers, Thromb Res 71:337–359, 1993 with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

illustrates an example of a pedigree of one of the seven kindreds studied showing the influence of co-existing APC-R and homocysteinemia on venous thrombosis. These authors recommended that patients with homocysteinemia and thrombotic events should be further evaluated for other inherited thrombotic disorders, especially APC-R [29].

Clinical Considerations

Elevated levels of plasma homocysteine, regardless of the underlying etiology, may be efficiently reduced in the vast majority of patients by dietary supplements of vitamins involved in methionine metabolism: B_6, B_{12}, and folic acid [4]. The importance of folic acid is emphasized by a statistically significant inverse relationship between the serum folate level and the risk of fatal coronary heart disease [30]. Boushey et al. [31] propose that folic acid supplementation may help prevent premature atherosclerotic vascular disease. Similarly, in subjects with premature atherosclerosis who are mildly homocysteinemic, Franken et al. [32] recommend that these patients can be safely treated with vitamin supplements. Clinical trials are necessary to prove that lower levels of plasma homocysteine due to vitamin supplementation will result in reduced incidence of venous thrombosis [4] and premature atherosclerotic vascular disease [31].

PROTEIN C, PROTEIN S, AND ANTITHROMBIN III DEFICIENCIES

Protein C, protein S, and AT III deficiencies have traditionally been associated with venous thrombosis, together representing approximately 10–15% of inherited thrombosis [33]. Recently, Koster et al. [34] reported that reduced levels of protein S were not associated with venous thrombosis among 474 Leiden Thrombophilia Study patients with a first objectively documented thrombosis. While in contrast to many previous reports linking protein S deficiency and thrombosis, these authors suggested that additional genetic defects co-segregating with protein S deficiency may be responsible for the thrombotic tendencies reported. Clinical aspects of protein C and S deficiencies and AT III deficiency have been previously described [33].

LABORATORY EVALUATION

Appropriate Patient Group for Study

Appropriate candidates for inherited thrombotic studies should be under the age of 45 years at the time of the first thrombotic episode and have a positive family history for thrombosis. Acquired causes for thrombosis should be excluded, including vascular disease, malignancy, myeloproliferative disorders, and antiphospholipid antibod-

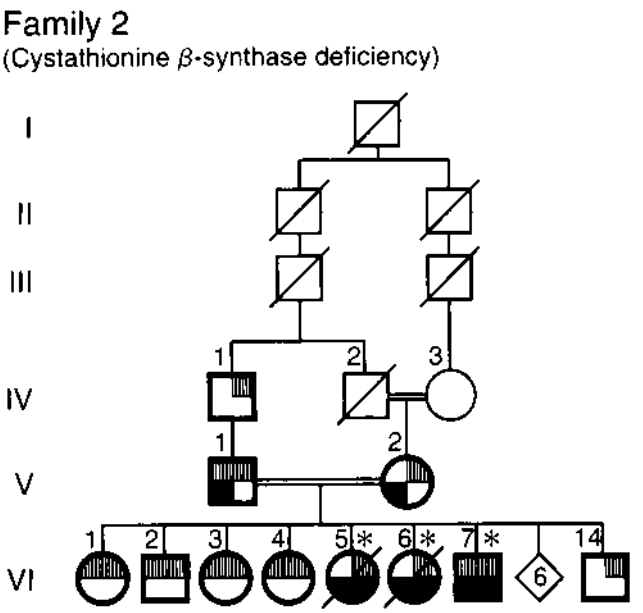

Fig. 2. Pedigree of a kindred with co-existing homocysteinemia and APC-R. Squares represent males; circles represent females. A slash through the symbol indicates death. Gray shading indicates APC-R (1/4 = heterozygote, 1/2 = homozygote); black shading indicates homocysteinemia (1/4 = heterozygote, 1/2 = homozygote). Asterisks indicate patients experiencing thrombosis. The diamond symbol indicates the number of siblings not studied. Reproduced from Mandell et al., N Engl J Med 334:763–768, 1996, Massachusetts Medical Society, all rights reserved.

ies [33]. Dizon-Townson et al. [35] report that among thirty women with the antiphospholipid syndrome, none were heterozygous or homozygous for the factor V Leiden mutation. The coexistence of APC-R with other acquired thrombotic disorders has not been studied, to our knowledge. However, patients with acquired thrombotic disorders and a positive family history for thrombosis should be considered for such testing.

APC-R is the most common cause for inherited thrombosis and is ten times more common than deficiencies of protein C, protein S, or AT III. Even though an understanding of APC-R is increasing rapidly, one study reported that testing for APC-R is ordered far less frequently than tests for other inherited thrombotic disorders such as AT III, protein C, and protein S deficiencies [17]. This data suggests that routine testing for APC-R may not be available to many physicians or that clinical pathologists or practicing physicians are unaware of this diagnostic possibility.

Sample Collection

Optimal laboratory evaluation is critically dependent on specimen quality. For plasma-based assays (functional and antigenic protein assays), venous blood should be collected by sterile venipuncture with a needle larger than 22 gauge, initially using a red-top vacuum tube (also known as a clot tube or pilot tube). Next, blood is collected into vacuum tubes containing 3.2 or 3.8% buffered sodium citrate. The blood is then centrifuged at 1,700*g* for 15 min and the top two-thirds of the platelet-poor plasma is then transferred to properly labeled plastic vials using a plastic transfer pipette. Some authors advocate 0.2 μm filtration of the platelet-poor plasma to remove any remaining platelets and platelet fragments to decrease the risk of falsely low APC-R results. Plasma should not be frozen prior to APC-R studies and testing should take place within 24 hr of collection. If the plasma must be frozen prior to shipment to a reference laboratory, the plasma should be passed through a 0.2 μm filter prior to freezing [36].

Homocysteine levels should be measured in plasma or serum because whole blood measurements can vary significantly (up to 180% of in vivo levels), due to cellular metabolism of L-methionine to homocysteine [37]. Falsely elevated homocysteine levels can be avoided by placing the blood sample on ice immediately after collection and preparing plasma or serum within 1 hr after collection. Homocysteine is stable at room temperature in plasma or serum samples for at least 4 days, longer if refrigerated (0–2°C) or frozen [38]. Food consumption may affect the plasma homocysteine concentration, causing falsely low [37] or falsely high values [38], so patients should not eat at least 8 hr prior to testing [37].

APC-R Testing

Functional assays are based on prolongation of the activated partial thromboplastin time (aPTT) in the presence and absence of exogenous APC, and expressed as the ratio: aPTT + APC/APTT without APC. If APC-R is not present, addition of APC will degrade factors Va and VIIIa, prolonging the aPTT by at least twofold. Likewise, if the mutated factor V is present, APC will not degrade the abnormal Va, and the molecule can participate normally in the generation of a fibrin clot with less prolongation of the aPTT. With properly collected samples, a normalized APC ratio test can identify 99.8% of these patients [39]. However, because these are aPTT-based assays, they are sensitive to anticoagulants, severe liver disease, lupus anticoagulants, etc. Samples are commonly submitted, inappropriately, with prolonged PT and/or PTT values, with one study reporting that 40% of its submitted specimens were not evaluable for functional APC-R testing for these reasons [17]. The specificity of this test can be improved by diluting the patient's sample with factor V-deficient plasma [40]. This test can also be used in patients on oral anticoagulants [40–42] and can eliminate the falsely low pregnancy-induced effect on the normalized aPTT ratio [43]. It should be noted that APC-R may cause false-positive protein S test results, if clotting

(functional) assays are used to diagnose protein S deficiency. It can be argued, therefore, that protein S-deficient patients diagnosed with a functional assay should also be tested for APC-R [44].

Some authors argue that functional assays alone are insufficient for diagnosis because (1) there can be considerable overlap in test results in patients with and without the mutation [45], (2) the observation that some patients with a low functional APC-R test do not show the factor V mutation, suggesting other factor V mutations or mutations in factor VIII, which have not yet been described [13,45], and (3) inappropriate sample submission for functional assays [17]. Consequently, an argument can be made that functional assays should be confirmed with the DNA test, or that the DNA test alone be performed.

Molecular Diagnostics

Of the inherited thrombotic disorders, APC-R appears to be an ideal candidate for routine polymerase chain reaction (PCR)-based analyses due to the highly conserved point mutation in factor V present in the vast majority of APC-R patients [6]. This is in contrast to the genotypically heterogeneous mutations resulting in protein C, protein S, and AT III deficiencies, which can require tedious exon-by-exon mutation screen analysis.

Several PCR-based assays have been employed in the diagnosis of APC-R. Techniques such as allele-specific amplification [46], single strand conformational polymorphism (SSCP or SSP) [47], and restriction endonuclease digestion of a specific PCR product have been described [6]; however, the exact role for these techniques in the diagnosis of APC-R is debatable. An obvious application for such tests is in anticoagulated patients, however, heparin has been reported to inhibit PCR [48], although Burckhardt [49] reported that heparinized whole blood samples are well-suited for DNA PCR with Taq polymerase. Our reference laboratory rejects blood samples mixed in vitro with heparin, but the blood levels seen with its use in vivo as an anticoagulant apparently have no effect on the PCR-based factor V Leiden test.

Homocysteinemia Testing

Several diverse methodologies are employed in the diagnosis of homocysteinemia. The most sensitive test appears to be serum homocysteine measurements before and after oral methionine loading [26,50]. Initial results from the NHLBI Family Heart Study [50] show that more than 40% of patients with clinically important homocysteinemia are missed by assaying a fasting total plasma homocysteine level alone. We reserve the use of methionine-loading tests for patients deemed to have an inherited disorder, and in whom tests for APC-R, protein C, protein S, and AT III deficiencies are normal.

Plasma homocysteine levels can be measured in a clinical chemistry reference laboratory by several methods, including high performance liquid chromatography (HPLC) quantitation [51], fluorescence polarization immunoassay [52], and others. Methodologies to measure total plasma homocysteine are reviewed in detail by Ueland et al. [38].

Molecular Diagnostics

A common point mutation in the MTHFR gene, 677C→T, is present in its homozygous form in 15% of a Dutch population with premature coronary artery disease (5% controls) [25]. This homozygous mutation is associated with an approximate threefold increase in premature cardiovascular disease [25]. CBS deficiency appears to be more genetically diverse with at least 17 documented mutations in homozygous CBS-deficient patients [53].

SUMMARY

A previous review of inherited thrombosis recommended sequential test ordering of functional assays until a specific diagnosis was achieved [33]. However, the emerging concept of multiple genetic risk factors being associated with a greater thrombotic potential would argue that all patients should be evaluated for multiple inherited disorders. Additionally, the confounding effects of protein S deficiency and APC-R on functional assay results [44] support the suggestion of extensive testing to precisely identify all genetic risk factors. If appropriate (non-anticoagulated) specimens cannot reliably be obtained, APC-R should be exclusively evaluated using DNA methods.

Despite the appreciation of the association of APC-R and homocysteinemia with inherited thrombosis, a diagnosis will not be made in up to 30% of patients with recurrent thrombosis, suggesting that additional genetic etiologies remain to be discovered. Nevertheless, the substantial advances in this area over the past few years have provided important information on the laboratory evaluation and management of these patients.

ACKNOWLEDGMENTS

We thank Steve M. Skoumal, M.D., J.D., for his review of this manuscript. G.M.R. is supported by funds from the VA Research Office.

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